



Comparison of safety, delivery, and efficacy of two oncolytic herpes viruses (G207 and NV1020) for peritoneal cancer

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G207 and NV1020 are two replication-competent, multiantigen oncolytic herpes simplex viruses evaluated in the current studies for their anticancer effects in the treatment of gastric cancer. Deletion of both $\gamma_134.5$ genes and inactivation of ICP6 (ribonucleotide reductase) allows G207 to selectively replicate within tumor cells. NV1020 is another attenuated recombinant herpes virus with deletions of the HSV joint region, with deletion of only one copy of the $\gamma_134.5$ gene, and with the ICP6 gene intact. *In vitro*, both G207 and NV1020 effectively infected, replicated, and killed human gastric cancer cells, with NV1020 being more effective at lower concentrations of virus. In a murine xenograft model of peritoneally disseminated gastric cancer, both NV1020 and G207 reduced tumor burden when given intraperitoneally (i.p.) at higher doses. When viral doses were lowered or when advanced tumor was treated, i.p. NV1020 was superior to i.p. G207. *In vitro* viral replication and cytotoxicity predicted the *in vivo* antitumor response. Intravenous delivery of either G207 or NV1020 failed to reduce tumor burden, demonstrating the importance of regional therapy as treatment for compartmentalized malignancy. Both agents were safe for use in animals, and immunohistochemistry performed on mouse tissue revealed selective viral targeting of tumor. Oncolytic therapy using genetically engineered HSVs represents a promising strategy for peritoneal malignancies.

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Identification and manipulation of strategic viral genes has generated herpes simplex virus type 1 mutants that can selectively kill cancer cells. Replication-conditional mutants have been created by deletion of genes coding for enzymes required for nucleotide synthesis such as thymidine kinase, ribonucleotide reductase (RR), or uracil *N*-glycosylase.^{1–6} Other recombinants have the $\gamma_134.5$ gene deleted, which attenuates neurovirulence while permitting viral growth in tumor cells.^{7,8} These oncolytic, single-gene mutants have effectively killed tumors in many experimental models, yet they all maintain the potential risk of reversion to the wild-type strain. In the current study, two second-generation vectors are evaluated that contain mutations of several viral genes, markedly decreasing the chances of wild-type reversion.^{9–11} G207 contains deletions of both $\gamma_134.5$ genes and has the ICP6 gene inactivated, which codes for RR.^{9,10} In preclinical studies,

this oncolytic virus has shown antitumor efficacy against a wide variety of solid malignancies, including neurologic, colorectal, head and neck, gastric, prostate, hepatocellular, lung, and breast cancers, and is currently being evaluated in clinical trials.^{10,12–18} NV1020 is a multiantigen HSV-1 originally designed as a vaccine against herpes simplex virus 1 and 2 infection¹⁹ that has recently been investigated as a candidate therapy for cancer.^{20,21} The HSV joint region has been deleted in NV1020 to attenuate the virus.¹⁹ Similar to other multiantigen HSVs, NV1020 selectively replicates within tumor cells.^{20,21} Compared to G207, the NV1020 virus maintains endogenous ribonucleotide reductase and one copy of the $\gamma_134.5$ neurovirulence gene.¹⁹

A number of factors contribute to the dismal prognosis of gastric cancer. Most patients have disseminated disease at the time of diagnosis and consequently cannot undergo curative surgical resection. There is a high rate of recurrence in those patients where resection is possible.^{22–24} Available chemotherapeutic agents have only limited activity on these tumors. Novel effective strategies to treat gastric cancer are therefore important clinically. We previously demonstrated that intraperitoneal (i.p.) G207 therapy could reduce tumor burden in animals with gastric carcinomatosis. The purpose of the

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current study was to evaluate the ability of i.p. NV1020 to treat peritoneally disseminated gastric cancer, and to compare both the efficacy and safety of NV1020 to G207. The importance of route of delivery was also evaluated by comparing i.p. and intravenous (i.v.) viral administration.

Materials and methods

Cells and cell culture

Three human gastric cancer cell lines were used for this study. MKN-45-P cells were supplied as a generous gift from Dr Yutaka Yonemura at Kanazawa University, Japan, and maintained in RPMI 1640. OCUM-2MD3 cells were supplied as a generous gift from Dr Masakazu Yashiro at Osaka City University Medical School, and were maintained in DMEM with high glucose, 2 mM L-glutamine, and 0.5 mM sodium pyruvate. MKN-74 cells were supplied as a generous gift from Dr Tetsuro Kubota at Keio University, Japan, and were maintained in RPMI 1640. All media contained 10% fetal calf serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained in a 5% CO₂ humidified incubator at 37 °C.

Viruses

G207 is an engineered herpes simplex virus based on the wild-type HSV-1 strain-F, and was received as a gift from DJ Rabkin and RL Martuza. As previously described, both $\gamma_134.5$ genes have been deleted, and the *Escherichia coli lacZ* marker gene has been inserted into the *UL39* gene, inactivating *RR₁₀₂₅*. NV1020 is a nonselected clonal derivative of R7020, an attenuated, replication-competent virus based on the HSV-1 strain-F, originally obtained from B Roizman.^{19,26} It has a 15-kb deletion over the joint region of the HSV-1 genome. This deletion encompasses the region of the genome coding for the ICP0, ICP4, latency associated transcripts (LAT), and one copy of the neurovirulence gene ($\gamma_134.5$). These deletions greatly attenuate virulence of the virus. Because it was originally derived as a vaccine against HSV-1 and HSV-2 infection,^{19,27} a fragment of HSV-2 DNA from the *HindIII* region encoding for several glycoprotein genes was inserted into the deleted joint region. It also has a 700-bp deletion of the endogenous *TK* locus that prevents expression of the overlapping transcripts belonging to the *UL24* gene. An exogenous copy of the HSV-1 *TK* gene was inserted under control of the $\alpha 4$ promoter. Both G207 and NV1020 were propagated on Vero cells and titered by standard plaque assay.

In vitro cytotoxicity assays

Previous studies have demonstrated varying levels of sensitivity to G207 replication and cytotoxicity among several different human gastric cancer cell lines.¹⁵ The MKN-45-P cell line is more resistant to cell kill by G207, followed by the OCUM-2MD3 and MKN-74 cell lines, which are more sensitive. These cell lines were used *in vitro* to evaluate the cytotoxic effects of NV1020 compared with G207. Cells were plated at 5×10^4 cells/well in 12-well plates (Costar, Corning, Corning, NY) and infected with either G207 or NV1020 at multiplicities of infection (MOIs)

of 0, 0.01, 0.1, and 1. Cell viability was counted at 24-hour intervals postinfection by trypan blue exclusion.

In vitro viral proliferation

Viral growth curves were performed to compare the ability of G207 and NV1020 to replicate in each of three human gastric cancer cell lines. Cells were plated at 5×10^4 cells/well in 12-well plates and infected with either G207 or NV1020 at an MOI of 0.01 (500 plaque-forming units [PFU]). This MOI was chosen to decrease the direct cytotoxic effects of the viruses and to measure production of viral progeny. Cells and supernatants were harvested at 24-hour intervals postinfection over 168 hours. Three cycles of freeze-thaw lysis were performed and viral titers were determined by standard plaque assay using Vero cells.

Animal studies

Athymic 4- to 6-week-old male mice purchased from the National Cancer Institute (Bethesda, MD) were used for all animal experiments. All animal work was approved by the Memorial Sloan-Kettering Institutional Animal Care and Use Committee and performed under strict guidelines. Animals were anesthetized by methoxyflurane inhalation for experimental procedures.

Establishment of peritoneally disseminated gastric cancer

Intraperitoneal injection of either OCUM-2MD3 human gastric cancer cells or MKN-45-P human gastric cancer cells results in peritoneally disseminated tumors, ascites, and cachexia, in well-established murine xenograft models.^{28,29} Intraperitoneal injection of 2×10^6 cells of either cell line reliably develops macroscopic nodules within 3 days, with significant tumor burden and abdominal distention occurring between 3 and 4 weeks. This i.p. dose of 2×10^6 cells/mouse was used for all animal experiments. Animal weights were recorded periodically to evaluate the effects of both tumor growth and viral therapy.

Treatment of gastric carcinomatosis with regional viral therapy

Several *in vivo* experiments were performed to compare the ability of G207 and NV1020 to reduce peritoneal tumor burden caused by two human gastric cancer cell lines. In the first set of experiments, 50 athymic mice were injected with OCUM-2MD3 cells and another 50 were injected with MKN-45-P cells. Three days after tumor inoculation, animals from each cell line were divided into five groups ($n=10$ /group) and treated with i.p. injection of either 5×10^6 PFU (medium dose) of G207 or NV1020, or with 5×10^7 PFU (high dose) of G207 or NV1020. In subsequent experiments, animals inoculated with OCUM-2MD3 or MKN-45-P cells were treated with lower dose viral therapy of either 5×10^5 PFU ($n=9$ /group) or 2.5×10^6 PFU ($n=9$ /group), respectively. Control animals were treated with i.p. injection of serum-free media. Animals were sacrificed 3 weeks after initial tumor challenge and peritoneal tumor burden was assessed by weight. At sacrifice, all abdominal organs with associated peritoneum were removed *en bloc*. The mesentery, diaphragm, omentum, gonadal fat, and all

associated tumor were systematically stripped from the bowel and associated organs and weighed as peritoneal tumor specimens. The peritoneum was also harvested from mice naive to both tumor and virus to establish baseline peritoneal weight. Tumor weight was determined by subtracting baseline peritoneal weight from total peritoneal and tumor weight. To further evaluate a model of more advanced tumor burden, animals inoculated with OCUM-2MD3 cells were treated 7 days later with either 5×10^7 PFU of G207 or NV1020 ($n=9$ /group), given by i.p. injection. For all experiments, animals were sacrificed 3 weeks after tumor challenge and peritoneal tumor burden was assessed by weight as described.

Treatment of gastric carcinomatosis with systemic viral therapy

The ability of systemic viral delivery to reduce peritoneal tumor burden was evaluated by treating animals with single dose and multidose i.v. regimens. Fifty animals were inoculated with OCUM-2MD3 cells and treated by tail vein delivery of virus (or media). Two groups were given either 5×10^7 PFU of G207 or NV1020 as a single tail vein injection ($n=9$ /group). Another two groups were treated with three i.v. doses of 5×10^7 PFU of G207 or NV1020, with the first injection given 3 days after tumor inoculation, and the other two i.v. injections given every other day ($n=10$ /group). A control group ($n=10$) was treated with i.v. injection of serum-free media. Animals were sacrificed 3 weeks after tumor challenge and peritoneal tumor burden was assessed by weight as described. Animal weights were recorded periodically to evaluate systemic toxicity.

Evaluation of innate immunity to neutralize virus

Recent studies have shown that both rat and human sera may have the innate capacity to inhibit viral activity.³⁰ Because i.v. viral delivery was ineffective in reducing tumor burden (see Results), the possibility of viral neutralization by serum was further investigated. Blood was collected from several tumor-bearing mice ($n=4$) that had never been treated with virus and was allowed to clot. Sera were collected after centrifugation at 14,000 rpm \times 10 minutes and complement activity was maintained by keeping specimens at $\leq 37^\circ\text{C}$, because complement-rich serum has been shown to be more active in inhibiting virus than heat-inactivated, complement-depleted serum.³⁰ Fifty microliters of serum from each mouse was incubated at 37°C with 300 PFU of G207 in 50 μl of serum-free media, and separately with 300 PFU of NV1020 in 50 μl of serum-free media. Standard plaque assay on confluent Vero cell monolayers was performed with each 100- μl mixture. Control plaque assays were done in parallel with 300 PFU of G207 and NV1020 incubated without mouse sera.

Survival study after regional viral treatment of gastric carcinomatosis

The ability of G207 and NV1020 to prolong survival in a gastric carcinomatosis model was determined at several doses. Mice ($n=40$) injected i.p. with OCUM-2MD3 cells were divided into five groups ($n=8$ /group) and treated

3 days later. G207 or NV1020 was administered by i.p. injection at doses of either 5×10^5 PFU (low dose) or 5×10^6 PFU (medium dose). Control mice were treated with serum-free media. Animals were checked daily for survival.

Histopathologic evaluation of dissemination and toxicity

Organ specimens from animals treated with 5×10^7 PFU of G207 or NV1020 were analyzed by HSV immunohistochemistry and hematoxylin counterstaining. Brain, liver, kidney, and peritoneal tumor were harvested at sacrifice, placed fresh in 4% paraformaldehyde, fixed overnight, and subsequently embedded in paraffin. Eight-micrometer slices were cut on a microtome, fixed on glass slides, and stored at 4°C until staining. HSV immunohistochemistry (IHC) was performed to evaluate G207 and NV1020 infection and dissemination to both peritoneal tumor and to distant organs. A HistoMouse (Zymed, San Francisco, CA) kit was used with a primary rabbit polyclonal anti-HSV antibody (Biogenex, San Ramon, CA), according to the manufacturer's protocol. Control organs were harvested from tumor-bearing animals never infected with virus and were stained in parallel with viral treated specimens. All slides were read by an independent, experienced pathologist (SM).

Results

In vitro cytotoxicity assays

To examine and compare the oncolytic efficacy of NV1020 and G207, dose-dependent cytotoxicity assays were performed on three human gastric cancer cell lines. Previous studies have shown that MKN-45-P cells are more resistant to G207, whereas the OCUM-2MD3 and MKN-74 cells are more sensitive.¹⁵ By 144 hours postinfection in the MKN-45-P cell line, an MOI=0.01 for G207 killed only 3% of cells whereas NV1020 killed 28% of cells ($P=.05$) (data not shown). Using an MOI=0.1, G207 killed 38% of MKN-45-P cells and NV1020 killed 93% of cells ($P<.0001$) (Fig 1A). At an MOI=1, cell death reached 99% for G207 and 100% for NV1020 ($P<.01$) (Fig 1A). For the most sensitive cell lines, both G207 and NV1020 were highly effective at killing cancer and no difference can be detected. The MKN-74 cell line was the most sensitive to viral cell kill among the three cell lines assessed. At 144 hours postinfection using an MOI=0.01, G207 demonstrated 97% cytotoxicity and NV1020 showed 99% cytotoxicity ($P<.01$) (data not shown). An MOI=0.1 killed 99% of cells for both viruses, and an MOI=1 killed 100% of cells (Fig 1C). In the sensitive OCUM-2MD3 cell line there was also no difference in efficacy between these viruses at an MOI=0.01, with 23% and 28% cell kill for G207 and NV1020 at 144 hours, respectively (data not shown). At an MOI=0.1, G207 killed 70% of OCUM-2MD3 cells whereas NV1020 killed 91% of cells ($P<.0001$) (Fig 1B). At an MOI=1, both G207 and NV1020 killed 100% of cells (Fig 1B).

In vitro viral proliferation

Viral titers were determined over 168 hours for both G207 and NV1020 to determine cellular permissiveness to viral proliferation. An MOI=0.01 was used to evaluate sustained

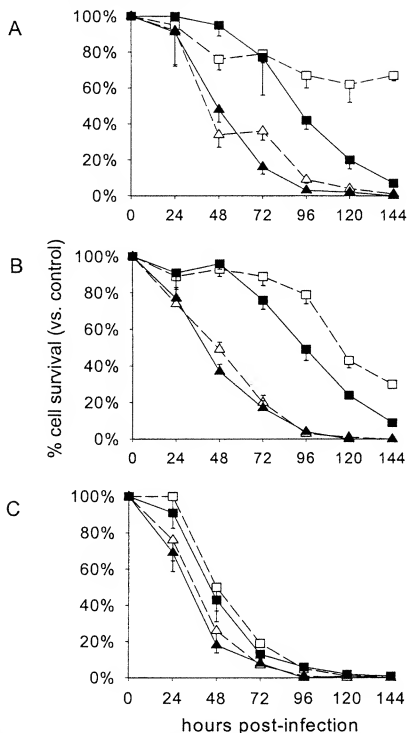


Figure 1 *In vitro* cytotoxicity of G207 and NV1020 against three human gastric cancer cell lines. Monolayer cell cultures were infected at MOIs of 0.1 (squares) and 1 (triangles) for (A) MKN-45-P cells, (B) OCUM-2MD3 cells, and (C) MKN-74 cells. Data for G207 are shown in open symbols, for NV1020 in filled symbols. Viable cells were counted every 24 hours by trypan blue exclusion, presented as mean cell survival (vs. controls) from triplicate wells (\pm SD).

proliferation at a less toxic viral titer. In the MKN-45-P cell line, NV1020 replicated exponentially greater than G207 ($P < .05$) (Fig 2A). By 168 hours postinfection, G207 was present in titers of 6.2×10^3 PFU, whereas NV1020 yields were 9.2×10^6 PFU ($P < .001$). In the OCUM-2MD3 cell

line, NV1020 proliferation was greater than G207 ($P < .05$) (Fig 2B). By 168 hours postinfection, G207 titers were 6.3×10^4 PFU, whereas NV1020 titers were 1×10^6 PFU ($P < .01$). In the MKN-74 cell line, NV1020 replication was higher than G207 from 48 to 96 hours postinfection

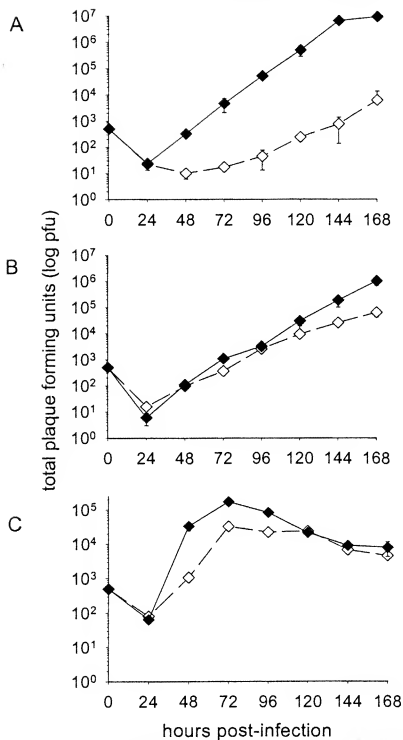


Figure 2 Replication of G207 (open) and NV1020 (filled) in three human gastric cancer cell lines. *In vitro* infection with a MOI of 0.01 (500 PFU) for (A) MKN-45-P cells, (B) OCUM-2MD3 cells, and (C) MKN-74 cells was performed, and virions were collected every 24 hours over 168 hours. Data represent mean PFU (\pm SD) from triplicate samples.

($P < .005$) (Fig 2C). Peak titers were collected at 72 hours for both viruses, with G207 reaching 3.3×10^4 PFU and NV1020 reaching 1.7×10^5 PFU ($P < .005$). Rapid and early viral proliferation in this cell line resulted in sufficient cell killing that led to a decrease in the number of viral particles assayed after 72 hours.

Treatment of gastric carcinomatosis with regional viral therapy

Tumor-bearing mice treated with regional delivery of either G207 or NV1020 showed a significant reduction in tumor burden when compared to control animals. The first efficacy

study compared both medium (5×10^6 PFU) and high (5×10^7 PFU) doses of G207 with NV1020, delivered 3 days after tumor inoculation by i.p. injection. For the experiment using OCUM-2MD3, peritoneal treatment with 5×10^7 PFU of virus reduced tumor burden, from 1380 ± 310 mg in controls to 250 ± 60 mg in the G207 group ($P < .01$) and

150 ± 40 mg in the NV1020 group ($P < .005$) (Fig 3A). Peritoneal treatment with 5×10^6 PFU reduced tumor burden to 360 ± 130 mg in the G207 group ($P = .01$ vs. controls), and 440 ± 170 mg in the NV1020 group ($P < .02$ vs. controls) (Fig 3A). There was no difference between treatment groups at these doses. For the experiment using MKN-45-P,

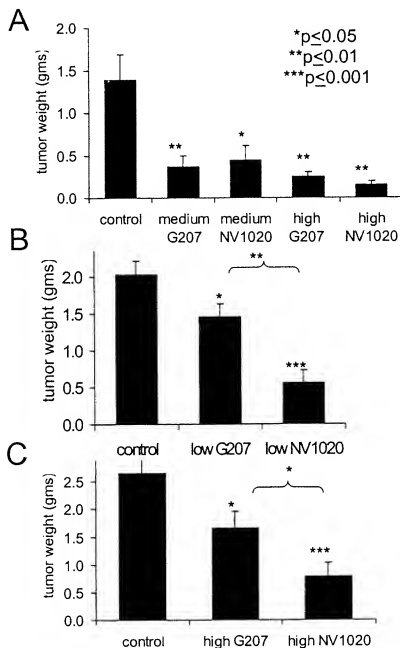


Figure 3 Comparison between G207 and NV1020 as intraperitoneal (i.p.) viral therapy for OCUM-2MD3 gastric carcinomatosis. Peritoneal OCUM-2MD3 gastric cancer cells were treated with G207 or NV1020, sacrificed 3 weeks later, and tumor burden was assessed by weight. **A:** Tumor bearing mice were treated 3 days post tumor inoculation with i.p. injection of 5×10^6 (medium dose) or 5×10^7 (high dose) PFU of either G207 or NV1020; control animals were treated with media. * $P \leq .05$ for medium NV1020; ** $P \leq .01$ for medium G207, high G207 and high NV1020. **B:** Mice inoculated with tumor were treated 3 days later with 5×10^6 (low dose) PFU of either G207 or NV1020. * $P \leq .05$ for low G207; *** $P \leq .001$ for low NV1020, and NV1020 was significantly better than G207 (** $P \leq .01$). **C:** Advanced tumor burden was treated by i.p. delivery of high-dose G207 or NV1020 given 7 days after tumor cell inoculation. * $P \leq .05$ for high G207; *** $P \leq .001$ for high NV1020, and NV1020 was significantly more effective than G207 (* $P \leq .05$). Statistical analysis was performed using Student's *t* test.

peritoneal treatment with 5×10^7 PFU of virus reduced tumor burden, from 1090 ± 110 mg in controls to 450 ± 80 mg in the G207 group ($P < .001$) and 540 ± 140 mg in the NV1020 group ($P < .01$) (Fig 4A). Peritoneal treatment with 5×10^6 PFU reduced tumor burden to 550 ± 70 mg in the G207 group ($P = .001$ vs. controls), and 540 ± 130 mg in the NV1020 group ($P < .01$ vs. controls) (Fig 4A).

Low-dose therapy was evaluated by treating OCUM-2MD3 and MKN-45-P carcinomatosis with 5×10^5 PFU of

either G207 or NV1020. In the OCUM-2MD3 tumor model, peritoneal delivery of 5×10^5 PFU of virus reduced tumor burden from 2030 ± 210 mg in controls to 1460 ± 210 mg in the G207 group ($P = .05$), and 550 ± 210 mg in the NV1020 group ($P < .001$) (Fig 3B). There was a significant difference in efficacy between NV1020 and G207 at these equivalent doses ($P < .01$) (Fig 3B). In the MKN-45-P tumor model, neither G207 nor NV1020 were effective at 5×10^5 PFU. Control tumors weighed 1320 ± 250 mg, whereas G207 and NV1020 tumors weighed 960 ± 150 and 940 ± 120 mg, respectively. When the medium dose was halved, i.p. treatment in the MKN-45-P model with 2.5×10^6 PFU resulted in tumors that weighed 1320 ± 250 mg for the control group, compared with 1070 ± 200 mg in the G207 group ($P = NS$) and 710 ± 150 mg in the NV1020 group ($P = .05$) (Fig 4B).

To simulate a scenario of delayed treatment, animals injected with OCUM-2MD3 cells were not treated until 7 days post tumor inoculation. Peritoneal tumor burden in the control group was 2660 ± 270 mg, which was reduced to 1650 ± 290 mg in the group treated with 5×10^7 PFU of G207 ($P < .05$) and 790 ± 230 mg in the group treated with 5×10^7 PFU of NV1020 (Fig 3C). There was a statistically significant difference between the G207- and NV1020-treated groups ($P < .05$) (Fig 3D).

Treatment of gastric carcinomatosis with systemic viral therapy

Route of delivery was studied to determine if i.v. viral administration was capable of treating experimental OCUM-2MD3 gastric carcinomatosis. Mice were treated with either single or multiple i.v. injections of high dose (5×10^7 PFU) G207 or NV1020, started 3 days after tumor inoculation. Neither virus was capable of reducing tumor burden, even after three doses of 5×10^7 PFU of G207 or NV1020 (data not shown). Animals did not show any signs of toxicity, even after multi-high-dose i.v. therapy, and continued to maintain or gain weight throughout the study.

Evaluation of innate immunity to neutralize virus

To determine if intravascular delivery of virus was neutralized by innate immune components in nude mice, sera from four tumor-bearing mice were incubated with known quantities of either G207 or NV1020. The number of plaques counted on Vero cells was the same for all viral samples incubated with mouse sera compared with the number of plaques produced by virus in media alone (data not shown). These data were produced from 1:1 sera to virus ratio, compared with the 1:8 dilution that neutralized virus in other studies.³⁰ These results indicate that serum was not responsible for inactivating viral particles in the blood, and do not explain the lack of i.v. efficacy.

Survival study after regional viral treatment of gastric carcinomatosis

A survival study was performed to determine if tumor reduction after treatment with peritoneal G207 or NV1020

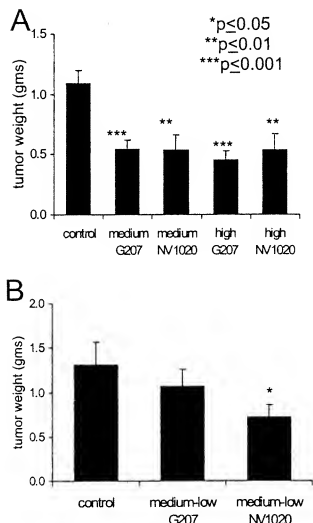


Figure 4 Comparison between G207 and NV1020 as i.p. viral therapy for MKN-45-P gastric carcinomatosis. Peritoneal MKN-45-P gastric cancer cells were treated i.p. with varying doses of either G207 or NV1020, sacrificed 3 weeks later, and tumor burden was assessed by weight. **A:** Tumor-bearing mice were treated 3 days post tumor inoculation with i.p. injection of 5×10^6 (medium dose) or 5×10^7 (high dose) PFU of either G207 or NV1020; control animals were treated with media. ** $P \leq .01$ for medium and high NV1020; *** $P \leq .001$ for medium and high G207. **B:** Mice inoculated with tumor were treated 3 days later with i.p. injection of 2.5×10^6 (medium-low dose) PFU of either G207 or NV1020. Only the NV1020-treated group showed a significant reduction in tumor burden when compared to controls. (* $P \leq .05$). Statistical analysis was performed using Student's *t* test.

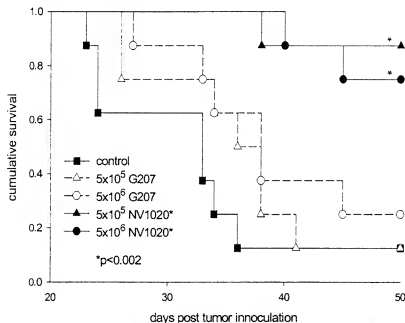


Figure 5 Extended survival after i.p. treatment of gastric carcinomatosis with NV1020. Mice inoculated with OCUM-2MD3 cells were treated 3 days later with i.p. injection of either 5×10^5 (low dose, triangle) or 5×10^6 (medium dose, circle) PFU of either G207 (open) or NV1020 (filled). Both doses of NV1020 were statistically different from both doses of G207 ($P < .03$ for all groups), or controls ($P < .002$). Statistical analysis was performed using Kaplan-Meier log-rank test.

conferred a survival advantage to mice. Mice inoculated with OCUM-2MD3 cells and treated i.p. with either low-dose (5×10^5 PFU) or medium-dose (5×10^6 PFU) NV1020 had a significant survival advantage when compared to controls (Fig 5). Median survival for control animals was 32 days, with 7 of 8 mice dead by 50 days. In the low-dose NV1020 group median survival was 49 days, with 1 of 8 mice dead by 50 days ($P < .001$ vs. controls), whereas median survival for medium dose NV1020 was 48 days, with 2 of 8 mice dead by 50 days ($P < .002$). Neither G207 dose was able to show a significant survival advantage when compared to controls. Mean survival for the low-dose G207 group was 36 days, with 7 of 8 mice dead by day 50, whereas mean survival for the medium dose G207 group was 39 days, with 6 of 8 mice dead by day 50 ($P = \text{NS}$). Both doses of NV1020 were statistically different from both doses of G207 (Fig 5) ($P < .03$ for all groups). Statistical analysis was performed using Kaplan-Meier log-rank test.

Histopathologic evaluation of dissemination and toxicity

Peritoneal tumor, brain, liver, and kidneys were evaluated for HSV infection and dissemination, and for the presence of necrosis after high-dose, i.p. viral therapy. An independent, experienced pathologist performed histologic evaluation. Tumors from untreated animals did not show signs of HSV staining or necrosis (Fig 6A). Significant 3+ HSV staining was seen in both G207 and NV1020 tumor specimens, with extensive necrosis detected nearby positive HSV staining (Fig 6, B and C, respectively). There was no evidence of HSV staining or necrosis in brain, kidney, or liver specimens evaluated after high-dose

i.p. G207 or NV1020 therapy (Fig 6, D–F, NV1020 tissue shown only).

Discussion

Oncolytic HSVs maintain several biologic properties that make them promising vectors for the treatment of cancer, in particular as therapy for diffusely spread malignancy. The ability of these viruses to selectively replicate within tumor cells means that they do not have to be directly injected into a mass to produce an antitumor response.^{2,14,15,31,32} Furthermore, smaller initial doses can be delivered with the expectation that viral progeny will kill neighboring, uninfected cells.^{8,15,20,21,25,33} These characteristics are secondary to strategic deletions in nonessential herpes genes that attenuate virulence, yet still allow recombinants to replicate in tumor cells.^{3,8,10,34} Because single-gene mutants maintain a risk for wild-type reversion, multimitated viruses such as G207 and NV1020 have been generated to improve safety.^{9–11} The current study sought to determine the relative efficacy of these two viruses in the treatment of a common cancer. The results indicate that these mutated viruses maintain the ability to replicate within gastric cancer cells, and are safe and effective in treatment of peritoneal metastatic diseases.

A model of gastric carcinomatosis was chosen for this study because of its clinical relevance. Gastric cancer is one of the most common malignancies worldwide, with carcinomatosis accounting for much of the fatality.^{23,24} The diffuse nature of this malignancy limits surgical resection as a treatment option, and chemotherapy is associated with high complication rates and minimal patient

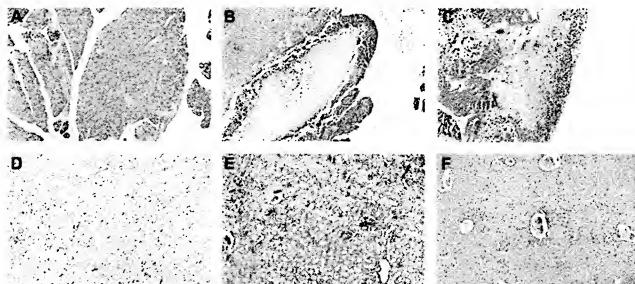


Figure 6 HSV immunohistochemistry demonstrates selective viral tumor targeting. Tissue specimens from animals treated i.p. with 5×10^7 (high dose) PFU of either G207 or NV1020 were paraffin embedded and stained for the presence of HSV antigen. **A:** Tumors from untreated mice showed negative staining and no signs of necrosis. Tumors from both G207-treated (**B**) and NV1020-treated (**C**) mice demonstrated strong (3+) staining for HSV and had adjacent areas of necrosis. Brain (**D**), kidney (**E**), and liver (**F**) tissue from viral treated animals showed negative staining for HSV (only NV1020 shown).

benefit.^{35,36} Patients with gastric cancer almost always present with peritoneal disease before progressing to systemic disease, indicating that the peritoneum is a possible target for novel therapies.^{23,24,36} The current data demonstrate promise for both HSV oncolytic viruses tested as regional therapy for such peritoneal disease. These data may also have implications for treatment of other malignancies that present with peritoneal dissemination, such as colorectal cancer, ovarian cancer, or pancreatic cancer.

The cellular basis for the differences in efficacy of G207 and NV1020 is likely multifactorial, including differences in RR, and $\gamma_{134.5}$. G207 contains deletions of both $\gamma_{134.5}$ neurovirulence genes and has a lacZ insertion mutation at the ICP6 locus, which inactivates RR.¹⁰ whereas NV1020 maintains one $\gamma_{134.5}$ gene and has RR intact.^{10,12,14} Viral growth of ICP6 mutants has been shown to occur only in cells that compensate for defects in viral RR and in cells that are rapidly dividing.^{4,5} In agreement with these findings, tumor cells with high proliferative indices have been the most susceptible to killing by RR mutants.^{32,37,38} The $\gamma_{134.5}$ gene product, ICP34.5, is also known to inhibit the protein kinase R (PKR) system, thereby preventing the shutdown of host protein synthesis and permitting viral replication.^{39,40} Viruses deleted for ICP34.5 are attenuated *in vivo* because the PKR system remains active and blocks protein synthesis and viral replication.^{41,42} A recent investigation has shown that wild-type replication and virulence of ICP34.5 mutants can be restored if PKR is deleted.⁴³ These studies suggest that only cells defective for PKR can permit significant replication of ICP34.5 mutants such as G207, thereby restricting the oncolytic potential of G207. Because cellular profiles within human tumors are heterogeneous, it is likely that clinical cancers harbor tumor cell populations with mixed PKR expression. It is likely that NV1020 could replicate better than G207 in these cells, given that NV1020

maintains endogenous RR. ICP34.5 expression in NV1020 may also permit viral replication within tumor cells independent of PKR status. Whether this is indeed seen in man awaits clinical investigation.

The current studies compared NV1020 and G207 in their ability to kill cancer cells *in vitro* and *in vivo*. At high doses, particularly in sensitive cell lines, both viruses were very effective in killing tumor cells and no differences can be discerned. At lower MOIs *in vitro*, both viral replication and cytotoxicity of NV1020 were greater than G207 for all three cell lines. These results paralleled the *in vivo* data seen after i.p. delivery of virus at lower viral titers, which demonstrated superior efficacy of NV1020 compared with G207. These initial studies were performed with viral delivery given 3 days after tumor inoculation, when small, macroscopic nodules were disseminated throughout the peritoneum. The superior clinical effect of NV1020 was even more evident in the studies using late viral delivery, where we modeled the patient with advanced cancer with more numerous and bulky tumors. Previous investigations using this peritoneal model demonstrated a survival advantage to mice treated with 5×10^7 PFU of G207.¹⁵ The current study therefore focused on survival after administration of lower viral doses and demonstrated that only i.p. NV1020 treatment could provide a survival advantage when compared to G207 at these titers. Thus, at lower doses, in particularly resistant tumors, or when therapy is directed at bulky tumors, NV1020 appears superior.

The safety of these multimutant viruses has been well established in previous studies.^{9,11,13–15,44} G207 has been directly injected into the brains of *Aotus* monkeys with no toxic side effects, and when delivered similarly in human trials showed no major toxicity at titers above 10^9 PFU.^{13,44} NV1020 was also shown to be safe in *Aotus* monkeys when delivered systemically in doses several thousandfold greater

than the known lethal dose of the wild-type virus.²⁶ The basis for attenuation of NV1020 may be secondary to deletion of the internal repeated region, and deletions of the UL5/6 gene and the L/S junction.^{19,26,45} In the current study, i.p. G207 and NV1020 were well tolerated in all animals. The specificity of tumor infection was also demonstrated by immunohistochemistry after i.p. delivery to detect the presence of herpes virus *in vivo*. Histologic assessment of peripheral organs revealed no signs of inflammation or injury, whereas tumor tissue was characterized by viral presence and necrosis.

A recent study demonstrated that innate antiviral activity present in rat plasma could neutralize HSV1 viral efficiency when virus is delivered i.v.³⁰ Our findings demonstrated that such innate viral inactivation by serum did not occur in the athymic mice used for these experiments. Using a protocol similar to this previous study, complement-rich sera from several mice were incubated with known quantities of G207 or NV1020 and assayed for viral inhibition. There was no reduction in viral plaque counts for either virus. Significant quantities of i.v. virus therefore appear to be inaccessible to peritoneal tumor. This may be due to the inherently poor blood supply to the peritoneum, compared with other well-perfused organs where vascular delivery of oncolytic viruses have effectively killed tumor.^{12,14,32,38} Other studies have shown that blood flow in the microcirculation of peritoneally disseminated tumors is markedly reduced, thereby decreasing the bioavailability of systemically administered agents to peritoneal tumor.⁴⁰ Intraperitoneal viral delivery circumvents these problems by facilitating free communication between viral particles and tumor nodules. These data would therefore encourage clinical investigation of HSV oncolytic therapy for gastric cancer and in particular for peritoneal therapy of intra-abdominally disseminated gastric cancer.

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